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Single cell sorting identifies progenitor cell population from full thickness bovine articular cartilage

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SUMMARY

Objective: To date, no approved clinical intervention successfully prevents the progressive degradation of injured articular cartilage that leads to osteoarthritis (OA). Stem/progenitor cell populations within tissues of diarthrodial joint have shown their therapeutic potential in treating OA. However, this potential has not been fully realized due in part to the heterogeneity of these subpopulations. Characterization of clonal populations derived from a single cell may help identify more homogenous stem/progenitor populations within articular cartilage. Moreover, chondrogenic potential of clonal populations from different zones could be further examined to elucidate their differential roles in maintaining articular cartilage homeostasis.

Method: We combined Fluorescence-activated cell sorting (FACS) and clonogenicity screening to identify stem/progenitor cells cloned from single cells. High-efficiency colony-forming cells (HCCs) were isolated, and evaluated for stem/progenitor cell characteristics. HCCs were also isolated from different zones of articular cartilage. Their function was compared by lineage-specific gene expression, and differentiation potential.

Results: A difference in colony-forming efficiency was observed in terms of colony sizes. HCCs were highly clonogenic and multipotent, and overexpressed stem/progenitor cell markers. Also, proliferation and migration associated genes were over-expressed in HCCs. HCCs showed zonal differences with deep HCCs more chondrogenic and osteogenic than superficial HCCs.

Conclusion: Our approach is a simple yet practical way to identify homogeneous stem/progenitor cell populations with clonal origin. The discovery of progenitor cells demonstrates the intrinsic self-repairing potential of articular cartilage. Differences in differentiation potential may represent the distinct roles of superficial and deep zone stem/progenitor cells in the maintenance of articular cartilage homeostasis.

Introduction

Cartilage lesions are a fairly common problem in orthopedic practice. However, as an avascular and aneural tissue, articular cartilage has minimal intrinsic healing ability[1]. More often, most macroscopic cartilage lesions not only cause local tissue damage, but initiate whole joint progressive cartilage degeneration, which will ultimately leads to osteoarthritis (OA)[2,3]. Stem cell-based treatments have been explored for enhancing cartilage repair in degenerating joint for the past few years[4–6]. Evidence has emerged on the existence of MSCs-like cells from the synovium, articular cartilage, infrapatellar fat pat[7–9], and other tissues within articular joints. These cells can be primed towards chondrogenic differentiation both *in vitro* and *in vivo*, thus might represent possible candidates to maintain normal turnover of cartilage as well as to restore damaged cartilage upon joint lesions. Nevertheless, more complete understanding of their reparative behaviors is needed to further explore their therapeutic potential.

Adult mesenchymal stem cells (MSCs), or cartilage chondroprogenitors, are known to reside residing in hyaline tissue and have been shown to be highly clonogenic, multipotent, and chemotactic[10–12]. These tissue stem/progenitor cells are able to migrate towards local injury sites, where they proliferate and differentiated as needed to replace damaged tissue[13,14]. Unlike MSCs, which are

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able to differentiate into multiple tissue types in different organ systems, tissue progenitor/stem cells are typically only capable of generating limited tissue types for local tissue regeneration, especially the tissue of their origin. Stem/progenitor cells in articular cartilage are an example of the latter cell type, one that is able to undergo multi-lineage differentiation, but *in situ* and in normal physiological conditions is lineage restricted to differentiate into hyaline cartilage-producing chondrocytes.

Chondrogenic progenitor cells (CPCs) were first discovered by Dowthwaite *et al.*, who identified them to be a subpopulation of superficial zone cells for appositional growth of articular cartilage[15], which have enhanced affinity to fibronectin and highly expressed stem cell-associated factor Notch-1. Koelling *et al.* have also found CPCs in articular cartilage during later stages of human OA[16], these cells were highly migratory towards damaged cartilage tissue and repopulated in repair tissue. Grogan *et al.* later examined the distribution of stem cells markers (Notch-1, Stro-1, VCAM-1), and found inconsistency between stem-cell marker expression and stem cells distribution, thus concluded that these stem cell markers may not be useful to identify progenitors in cartilage. Some other studies also showed stem/progenitor cells overexpressed stem cell surface markers (CD105, CD166)[17] and were capable of Hoechst 33,342 dye exclusion as a side population, characteristic of stem cells[18]. Moreover, we previously found migrating CPCs strikingly proliferating on the articular surface post traumatic injuries in an *in vitro* bovine osteochondral explant impact model in response to multiple alarmins released by necrotic cells[19]. Another study also showed that injured bovine cartilage induces migration of Notch-1 positive cells to the surface of damaged region[20].

Despite the evidence that these cells might represent a putative cartilage progenitor cell maintaining the homeostasis of the articular joint, only a few studies thus far have identified a homogeneous single cell-derived clonal sub-population within the normal articular cartilage[21]. Full characterization of stem/progenitor cell potential requires the generation of genetically identical populations from a single progenitor[22]. Otherwise, the phenotypic "stemness" may actually result from a heterogeneous pool of cells with different origins. Williams *et al.* has demonstrated clonal cartilage progenitor cells have distinct phenotype from full-depth chondrocytes, as well as different telomerase activity[23]. In addition, where progenitors from articular cartilage normally reside within extracellular matrix is still not clear and worthy further investigation.

In the present study, we describe, for the first time, a single cell clonogenicity screening technique to identify progenitor cells in healthy articular cartilage. This technique allows isolation of progenitors from the superficial 1/3 as well as deep 2/3 of full thickness cartilage, with distinct differences in differentiation potency. Genetic and functional characteristics of the high-efficiency colony-forming cells (HCCs) reveal their similarities with adult stem/progenitor cells.

Materials and methods

Cartilage tissue harvesting and cell isolation

Fresh stifle joints from young adult cattle (15–24 months old) were obtained from a local abattoir (Bud's Custom Meats). Articular cartilage was harvested from the femur condyle using a 6 mm biopsy punch [Fig. 1(B)] and rinsed in Hank's Balanced Salt Solution (Invitrogen, California, USA) supplemented with 100 U/μl penicillin, 100 μg/ml streptomycin, and 2.5 μg/μl fungizone. Full thickness cartilage biopsy samples were minced into fine pieces and digested overnight with 0.25 mg/ml collagenase type 1 and pronase E (1:1) (Sigma–Aldrich, St. Louis, MO) dissolved in culture medium in a shaking incubator overnight (0.25 mg/ml each). When needed, a customized apparatus was used to separate the superficial 1/3

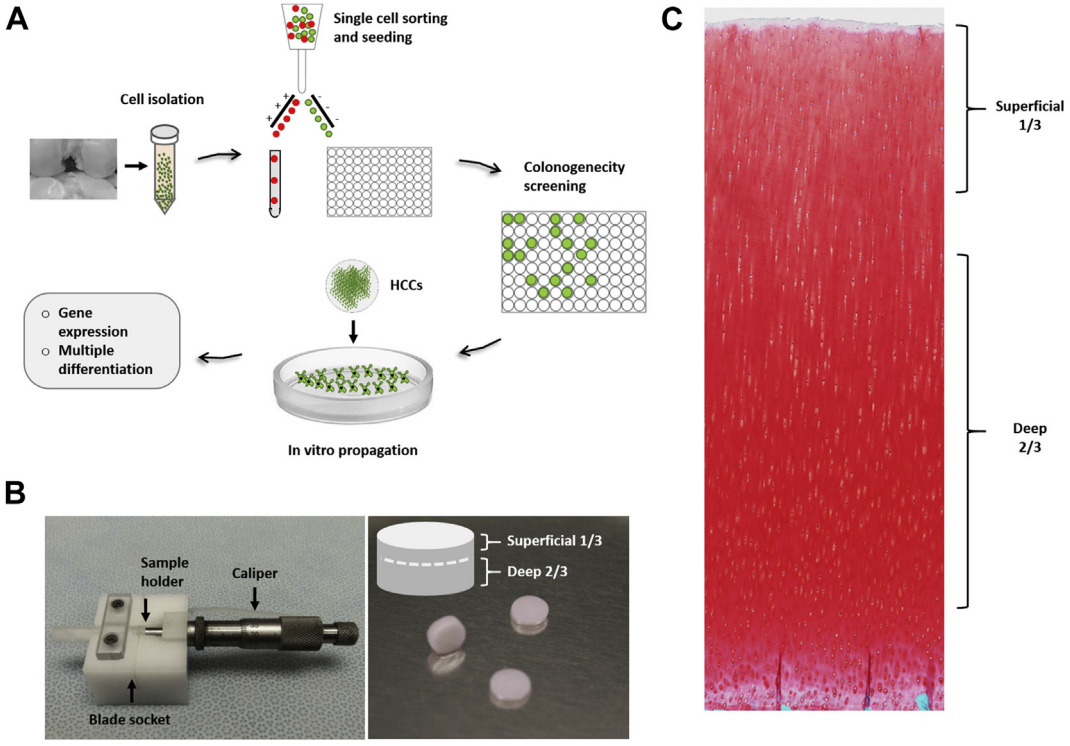


Fig. 1. Experimental schematics and customized apparatus. A) Flow diagram representing the methodology for isolating and characterizing cartilage progenitor cells. B) A customize-made apparatus for separating superficial and deep cartilage from 6 mm cartilage biopsy, with a caliper, and sample holder, and a blade socket. C) Histological image showing full thickness bovine articular cartilage (Scale bar represents 500 μm).

(including superficial zone) and deep 2/3 part of the cartilage biopsies prior to digestion [Fig. 1(B)]. The next day, the digestion solution was neutralized by culture medium and passed through different sized cell strainers (BD Falcon™, BD bioscience, Maryland, USA) serially (100 μm, 70 μm, and 30 μm) to obtain a single cell solution, which was confirmed by hemocytometer. $5–10 \times 10^6$ cells were suspended in 2 ml Hank's Balanced Salt Solution (Invitrogen, California, USA) in 5 ml Falcon Polystyrene Tube (BD bioscience, Maryland, USA) for fluorescence-activated cell sorting (FACS).

FACS and single cell plating

Prior to single cell sorting, 96-well culture plates were coated with 0.1% gelatin solution (Bio-Rad, CA, USA) to give optimal cell attachment. The single cell solution was subjected to 1 μg/ml Propidium iodide staining (Life technologies, NY, USA) for excluding dead cells during FACS (Becton Dickinson Aria II, BD, Maryland, USA). Viable cells were sorted into 96-well plates, one cell per well, sequentially, in Dulbecco's modified Eagle's medium (DMEM)-based culture medium [Fig. 1(A)]. The rest of the cells were re-plated and cultured in DMEM and Ham's F12 (1:1 mixture) supplemented with 10% KnockOut serum replacement (Life Technology, Grand Island, NY), 50 μg/μl L-ascorbate, 100 U/μl penicillin, 100 μg/ml streptomycin, and 2.5 μg/μl fungizone at 37°C with 5% CO_2.

Clonogenicity screening

After FACS and single cell plating, cells were cultured for 48 h at the same culture condition using DMEM-based medium. Beginning on day 3, culture wells were examined every other day under microscope to check the availability, location and size of colonies. At day 10, cultures were stained with 1 μg/ml Calcein-AM (green fluorescent for live cells). Colonies were analyzed based on green fluorescent detection using Olympus IX81 Inverted Light Microscope (Olympus, PA, USA). Colony sizes and numbers were measured by *ImageJ* according to the users' manual (rsb.info.nih.gov/ij). Colonies were categorized into confluent colonies (CCs), big colonies (BCs) that covered over ½ of the surface area of the well, and small colonies (SCs) according to their relative size [Fig. 1(A)].

Colony isolation and in vitro expansion

BCs and CCs were manually picked and passaged [Fig. 1(A)] serially to 24-well or 6-well culture plates (BD Bioscience, Maryland, USA) pre-coated with 0.1% gelatin solution (Bio-Rad, CA, USA). Cells were expanded in DMEM/F12 with GlutaMax (Life technologies, NY, USA) supplemented with 10% KnockOut serum replacement (Life Technology, Grand Island, NY), 50 μg/μl L-ascorbate, 100 μg/μl penicillin, 100 μg/ml streptomycin, and 2.5 μg/μl fungizone at 5% CO_2, 37°C. Media were replenished upon needed.

Immunofluorescence and immunocytochemistry staining

HCCs were isolated and seeded onto chamber slides for immunostaining. For Abcg2 (ab 3380, Abcam, Cambridge, MA) and Notch-1 (sc-6014, Santa Cruz Biotechnology, Inc., Dallas, TX), immunofluorescence staining was used. Primary antibodies was labeled at 1:400 and 1:200 dilution respectively, followed by Alexa 488 secondary antibody (Jackson Immunoresearch, West Grove, PA), and imaged by an Olympus FluoView™ FV1000 laser scanning confocal microscope (LSCM) (Olympus NDT Inc., MA). Lubrin staining was also performed on isolated HCCs from both superficial 1/3 and deep 2/3 cartilage using a mouse monoclonal antibody (ab 28484, Abcam, Cambridge, MA), and detected with and a Vectastain ABC kit (Vector, Burlingame, CA).

Table I
Primer information for PCR

	Forward	Reverse
ABCG2	CCTTGGTTGTCATGGCTTCA	AGTCCTGGGCAGAAGTTTTGTC
CD 105	CCACTGCCCCAGAGACTGCGC	GCCCCCACAGTGAGTGCTTAGGT
CD 90	CGGTGGTGTTTGGCCATGTAATGA	GAGAGAGGGGAGTCCTATCCTGGT
CD 73	AGCTTTCCCAGCCTTCCATGCG	GGGTGTCCTCTTGAGTCCTGCA
CD 29	GCGGCCTCCGGGTGGATTCC	GCCGGGAAGGTCCAGGGGC
RunX-2	GCATGAAGCCCTATCCAGAGTCT	GCTGATGGAGCTGTTGGTGTAG
Sox-9	CGGTGGTGTTTGGCCATGTAATGA	GAGAGAGGGGAGTCCTATCCTGGT
B-actin	TCGACACCGCAACCAGTTCGC	CATGCCGGAGCCGTTGTCGA
CXCL-12	AGATGCCCTTGCCGATTC	TCTTCAGCCTTGCCACGA
Dock-10	ATCCCAGTAGCAACGAGC	ATCATGTGGTCAGCGAAG

Gene expression analysis

For gene expression analysis, RNA was extracted directly from passage two BCs and CCs. Passage two normal chondrocytes (NCs), isolated from the same specimens and cultured for the same period of time, were used as controls. Cells were homogenized in TRIzol® reagent (Invitrogen™ Life Technologies, Carlsbad, CA) and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A previous study found that progenitor cells usually overexpress genes commonly expressed in MSCs (Mesenchymal stromal cells). Thus, markers examined were: ATP-binding cassette sub-family G member 2 (ABCG2), which is a characteristic gene of "side population" identified by flow cytometry to be stem/progenitor cells; Telomerase reverse transcriptase (TERT) gene is an indicator of length of telomeres, which is a genetic marker for both stem cells and cancer cells. Both of ABCG2 and TERT were previous shown to have increased expression in CPCs[24]. Sox-9 is a chondrogenic transcription factor, which is related to chondrogenic potential; RunX-2 is an osteogenic marker for progenitor cells. In addition, an array of clusters of differentiation (CD) markers (CD105, CD90, and CD133, *etc.*) commonly seen in stem/progenitor cells, were also examined. Moreover, PRG4 (lubricin encoding gene) was analyzed to compare its expression between HCCs from superficial 1/3 and deep 1/3. RT-PCR and qRT-PCR were used to compare the expression of these markers in NCs, BCs, and CCs, essentially as described[25,26]. Relative expression levels compared to the house-keeping gene were calculated using the e $2^{\Delta Ct}$ method. Primers were purchased from Integrated DNA Technologies (Coralville, IA). Table I summarizes the primers used in the PCR analysis.

Cell migration/chemotaxis assay

Cell migration/chemotaxis assays were performed using a CytoSelect 24-Well Cell Invasion Assay kit (Cell Biolabs) according to manufacturer's instructions. HCCs or NCs suspensions from full thickness cartilage (5×10^5 cells in serum-free medium (SF)) were added to the upper Transwell and placed in reservoirs containing SF alone or SF with 20 nM HMGB-1 (High-mobility group protein B1), a nuclear alarmin released by necrotic cells post injury. The plates were incubated for 24 h prior to processing. Cell lysates from the culture plate were then transferred to fluorescence plates and read on a micro-plate reader (Molecular Devices, California, USA). The data are presented as the relative fold-change regarding fluorescent intensity readings.

Multi-lineage differentiation assay

The multi-potency of HCCs (BCs and CCs) was examined by performing chondrogenic, osteogenic and adipogenic differentiation, respectively. For chondrogenic induction, 1.5×10^6 cells from

each group were pelleted in 15 ml conical tubes at 300 ×g for 5 min, and then cultured in chondrogenic medium (DMEM containing 10 ng/ml TGF-β1, 0.1 μM dexamethasone, 25 μg/ml L-ascorbate, 100 μg/ml pyruvate, 50 mg/ml ITS + Premix and antibiotics) at 5% CO_2, 37°C for 3 weeks. The resulting pellets were cryosectioned and analyzed for extra cellular matrix (ECM) formation by Safranin-O/fast green staining. For osteogenesis, HCCs were seeded into 12-well plates at 2×10^4 cells/well and cultured in osteogenic medium (DMEM/F-12 containing 0.1 μM dexamethasone, 100 mM β-glycerophosphate, 50 μg/ml L-ascorbate and antibiotics) 5% CO_2, 37°C for 3 weeks. Alizarin Red staining was used to detect calcium phosphate deposition. STEMPRO® Adipogenesis differentiation kit (GIBCO, Grand Island, NY) was used to induce adipogenesis according to the manufactures instructions. 3 weeks post-induction, cells were subjected to Oil Red O staining and imaged on a Nikon XB inverted microscope.

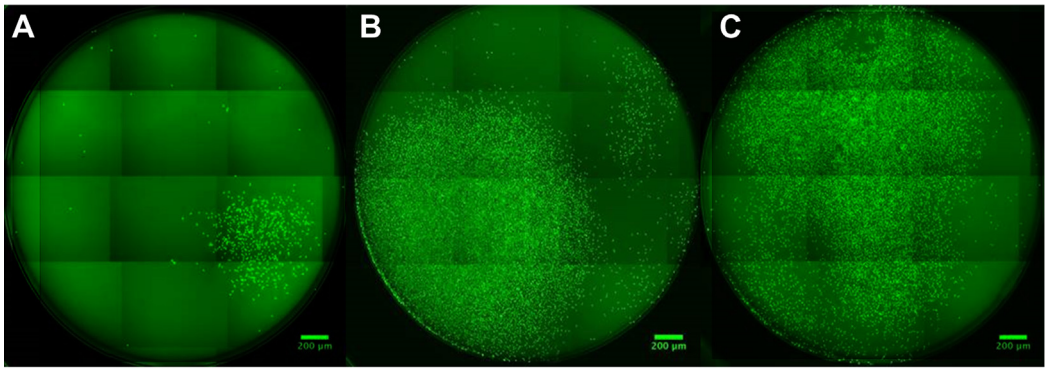
Statistical analysis

For clonogenicity screening, results were pooled from cartilage harvested from six animals with experiments done in duplicates. For gene expression studies, three different colonies for each group from each animal were tested. Statistical analysis was performed by Student's *t* test for each target gene. Migration assay was done by pooling HCCs from each animal ($n = 6$) and run in triplicates and Student's *t* test was used confirm statistical significance between each treatment group. All statistical analysis was completed by the GraphPad Prism software package (Lajolla, California, USA).

Results

Discovery of HCCs

The number of cells and the size of the colonies varied from each individual single cell as determined by comparing the images taken at different time points. Individual cells grew at different rates with some single cells forming very big colonies very rapidly, while others were only able to give rise to SCs or did not form colonies at all. Tiled fluorescent images of individual wells with different sized colonies at day 10 post initial single cell sorting are presented in Fig. 2. We found HCCs, which either reached confluence (>2/3 surface area, 5 ± 1%) [Fig. 2(C)], or had big-colony formation (1/3–2/3 surface area, 10 ± 2%) [Fig. 2(B)]. The percentage of HCCs was relatively small (about 15 ± 3%). Most of the cells (41 ± 4% of all cells) formed SCs (<1/3 surface area). HCCs started to form colonies (>50 cells) as early as day 5, unlike most cells, which showed only a limited number (<50) of cells growing at that time. Even at day 10, most of the wells had only small colony formation [Fig. 2(A)]; with



D

	Full thickness	Superficial	Deep
HCCs	15±3%	18±2%*	5±2%
Small Colony	41±4%	29±2%	38±2%
Non-colony	47±6%	52±1%	57±3%

E

	Cell number	Surface area percentage
Small	475± 78	17.2±4.3%
Big	8664±481	53.6±7.9%
Confluent	10974± 699	78.8±13.6%

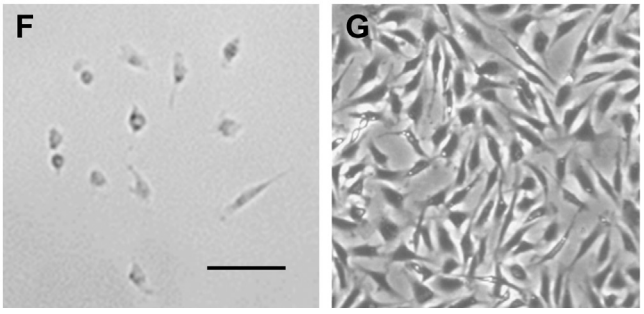


Fig. 2. Fluorescent images of different types of colonies and their relative ratio and dimension. A) Small colony. B) Big colony. C) Confluent colony. D) The percentage of different type of colonies from full thickness cartilage, and superficial 1/3 and deep 2/3, respectively. E) The criteria used for categorized colonies in regard to their size and cell number. (Scale bars = 200 microns) F) Non-colony forming cells with characteristic cobblestone-like shape of chondrocytes. G) CFCs displayed more stretched fibroblast-like morphology similar to mesenchymal stem cells. (Scale bar = 50 microns).

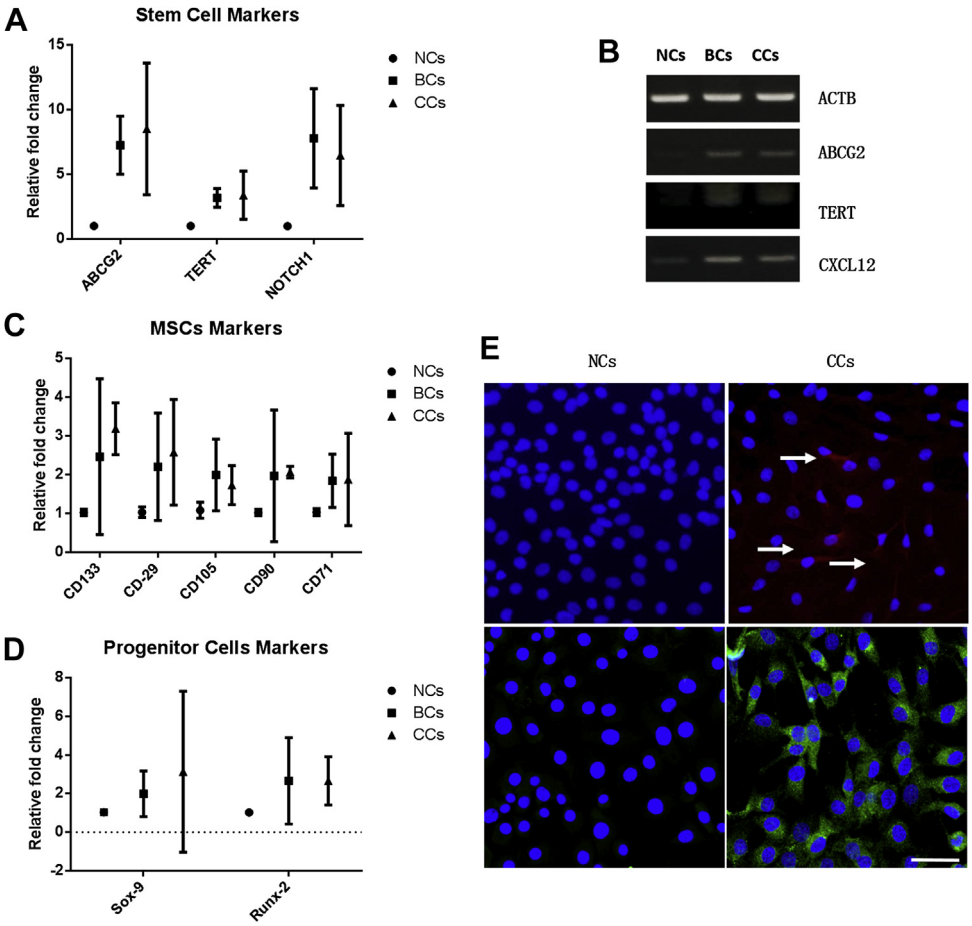


Fig. 3. Gene expression of HCCs (BCs and CCs). A, B) Real-time qRT-PCR and/or agarose gel electrophoresis showed significant overexpression of stem/progenitor cell makers ABCG2, TERT and Notch1 in BCs and CCs compared with NCs. C) A array of MSCs markers were significantly over-expressed level in BCs and CCs vs NCs. D) Higher expression of chondrogenic transcription factor SOX-9 and osteogenic transcription factor RUNX-2 in BCs and CCs relative to NCs. E) CCs showed positive staining for both ABCG2 (upper right) and Notch-1 (lower right) in comparison to NCs. (Scale bar represents 50 μm). The error bars in each plot represent the 95% confidence interval (CI).

47 ± 6% showing no colony formation, even after 14 days culture (data not shown). Some cells proliferated within the first couple days, but failed to continue growing; instead keeping the same cell numbers throughout prolonged culture. Figure 2(D) summarizes the average percentage of cells that formed each type of colony ($n = 6$). Figure 2(E) summarizes the average cell number and surface area percentage of the colonies. Besides colony-forming efficiency, noticeable morphological differences were also observed between HCCs and Non-HCCs. Most HCCs displayed a fibroblast-like morphology, with a stretched and flat shape [Fig. 2(H)], while non-HCCs showed the characteristic cobblestone-like shape of normal chondrocyte cultures [Fig. 2(G)].

HCCs were isolated from both the superficial 1/3 and the deep 2/3 articular cartilage. Even though the total number of colony-forming cells (CFCs) was not significantly different between the two sites, with the superficial 1/3 having 46 ± 4% and the deep 2/3 having 46 ± 1%, by day 10, the superficial 1/3 site produced more HCCs formation (18 ± 2%) than did the deep 2/3 site (5 ± 2%)[Fig. 2(D)].

Stem/progenitor cell marker expression

Gene expression analysis revealed substantially higher expression of stem/progenitor cell marker genes in the HCCs vs the NCs. qRT-PCR showed that ABCG2 was increased by over 8-fold, and TERT by 3.2-fold. Moreover, agarose gel electrophoresis showed much stronger bands for ABCG2 and TERT in HCCs compared with NCs [Fig. 3(A)]. Sox-9 was increased by 3-fold, RunX-2 by 2.5-fold [Fig. 3(D)] and CD105, CD90, CD71, CD29, were increase around 2-fold in the HCCs over the NCs [Fig. 3(C)]. HCCs from the deep 2/3 showed higher RUNX-2, SOX-9, and COL1A1, COL2A1 expression, than colonies from the superficial 1/3 [Fig. 5(A) and (B)]. In addition, gene expression also confirmed higher level of lubricin was expressed in HCCs from superficial 1/3 with over 3-fold increase compared with deep HCCs [Fig. 5(C)]. Using immunostaining analysis, we showed that HCCs from full thickness cartilage showed positive staining for both ABCG2 and Notch-1, in comparison to NCs, which mostly are negative for these two stem/progenitor cells markers [Fig. 3(E)]. Also, for lubricin expression, HCCs from superficial 1/3 showed relatively intense lubricin staining signal with dark color for the cytoplasm, while HCCs from the deep 1/3 has only mildly stained cytoplasm with much brighter color mainly from H&E counter staining [Fig. 5(C)].

Chemotactic cell migration

In general, HCCs were significantly more active in Transwell chemotaxis assay than NCs with stimulation of chemotactic factor ($P = 0.0003$). Upon HMGB1 stimulation, HCCs showed strongly increased cell migration ($P = 0.001$) than untreated, while NCs did not have significant response to HMGB1 ($P = 0.2$). Compared with NCs, although HCCs did not show significantly more active migration ability ($P = 0.06$), they responded more rigorously to HMBG1

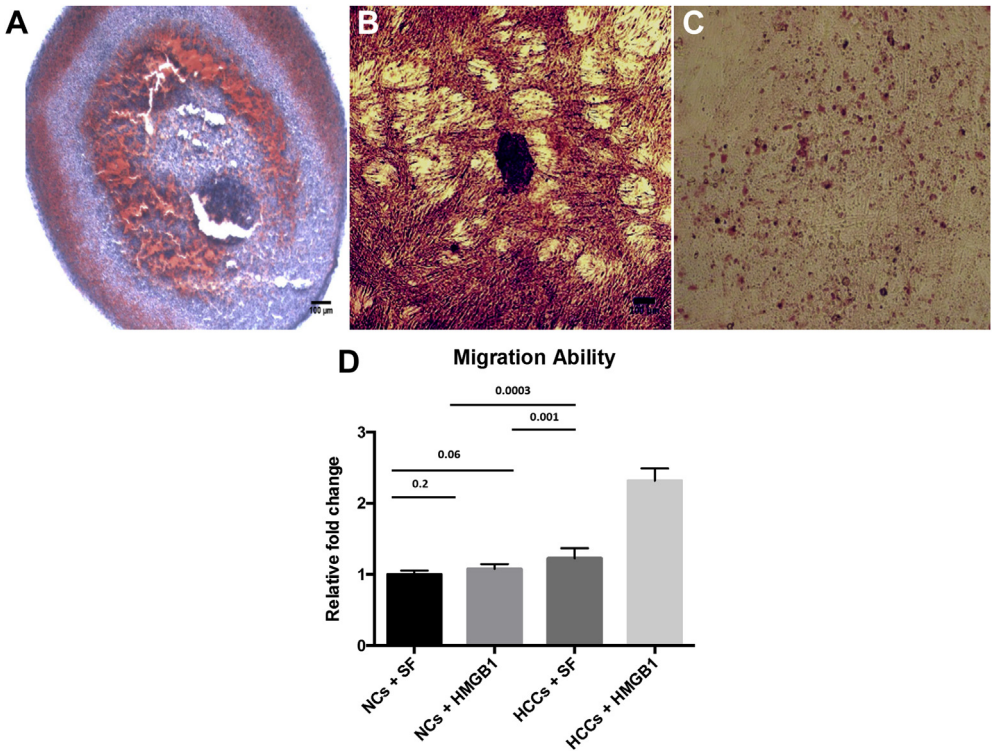


Fig. 4. Multi-lineage differentiation of HCCs and chemotactic migration A) Chondrogenic differentiation in pellet culture. Safranin-O/fast green staining displayed strong proteoglycan deposition. B) Osteogenic differentiation in monolayer cultured HCCs. Alizarin Red (dark red) staining showed massive calcium phosphate deposition. C) Adipogenic differentiation in monolayer HCCs. Only few cells were positive for Oil Red O staining. D) Migrating cells respond to HMGB1 stimulation. HCCs were strongly chemotactic to HMGB1, with significantly higher cell migration than treated with SF; HCCs also showed significantly higher migration than NCs upon HMGB1 stimulation. Numbers above the bars indicate *P* values for differences between each group; Bars show the mean ± 95% CI. (Scale bar represents 200 μm).

upon stimulation, with significantly increased migrating cells ($P = 0.003$) [Fig. 4(D)].

Multipotent differentiation ability of HCCs

Chondrogenic, osteogenic, or adipogenic induction was performed for HCCs in a 21-day culture period to evaluate their multilineage differentiation potential. Pellets from chondrogenic differentiation showed a substantial proteoglycan deposition throughout the histology section [Fig. 4(A)]. Similarly, HCCs cultured in osteogenic medium had some calcium phosphate deposition in extracellular matrix as detected by Alizarin Red staining [Fig. 4(B)]. However, very few cells were positive for Oil Red O staining after adipogenic induction [Fig. 4(C)]. Interestingly, HCCs from deep 2/3 showed higher potential for chondrogenicity, with stronger proteoglycan deposition in the pellets starting on day

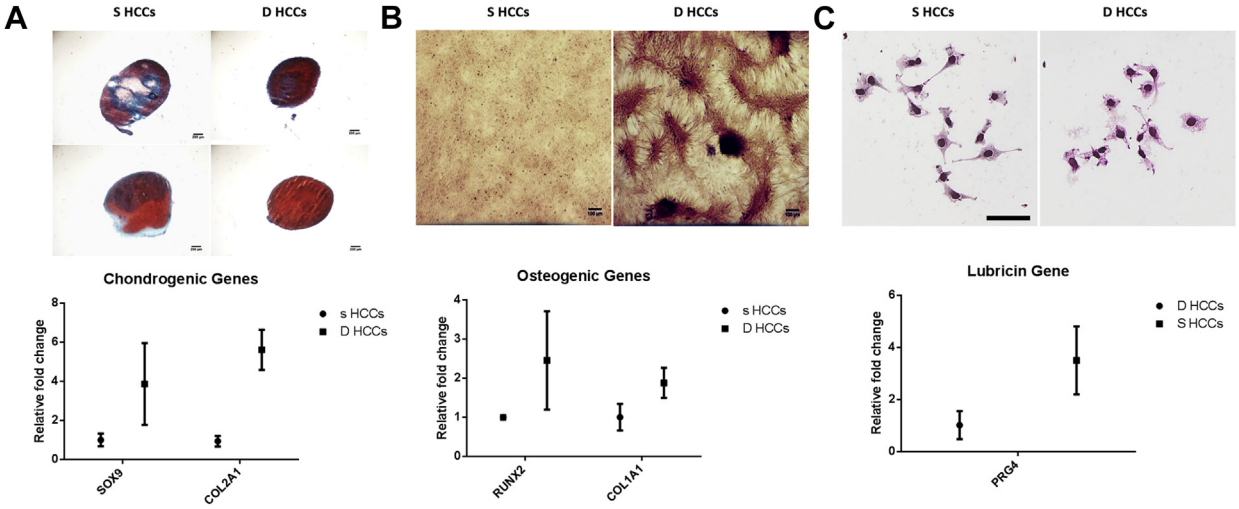


Fig. 5. Functional difference of superficial and deep HCCs. A) Deep HCCs showed superior chondrogenic potential with stronger Safranin-O staining for proteoglycan on both day 7 (left) and day 21 (right), and also showed higher chondrogenic genes expression. B) Deep HCCs had stronger osteogenic ability than superficial HCCs, with more calcium phosphate deposition on Alizarin Red staining and osteogenic markers higher expression. C) Superficial HCCs displayed higher level of lubricin both for immunostaining and gene expression than deep HCCs. The error bars in each plot represent the 95% CI.

7 [Fig. 5(A) upper right panel] through day 21 [Fig. 5(A) lower right panel] as compared to HCCs from superficial 1/3 [Fig. 5(A) lower panel]. HCCs from the deep 2/3 also showed substantially higher calcium phosphate deposition on Alizarin Red staining, with multiple calcium nodule formations, while HCCs from the superficial 1/3 had barely detectable staining signal for calcium phosphate [Fig. 5(B)], suggesting that these cells may be less differentiated.

Discussion

In this study, bovine chondrocytes are individually sorted into each well of the 96 well plates by FACS for viable cells, which allows the assessment of the clonogenic potential of each individual cell, thus enable us to isolate clonal population which might represent progenitor cells with the whole population. The HCCs cloned from a single cell closely resemble the progenitor cells identified in our previous study and by other groups, with the stem cell marker expression, clonogenicity, and multipotency consistent with published descriptions of progenitor cells from cartilage and other somatic tissues[23,27,28]. Differences of potency for HCCs from superficial 1/3 and deep 2/3 articular cartilage illustrate the distinct functions they may carry.

Clonogenicity screening confirmed the presence of CFCs within NCs isolated from full thickness articular cartilage. Based on differential colony-forming efficiency, we are able to identify a group of rapidly growing cells (HCCs) which either form big colonies or reach confluence in 96-well plates by day 10 post single cell seeding. Although nearly half of cells can form colonies, only very small portions of the cells are HCCs, whose active proliferating phenotype closely resembles transient amplifying progenitor cells.

High expression of stem cell markers like ABCG2 and TERT, strongly indicate that HCCs may represent a highly self-replicating progenitor cell population within cartilage. The high expression of a key chondrogenic transcription factor Sox-9 and an osteogenic transcription factor RunX-2, together demonstrated the possible bipotency of HCCs for differentiation towards these two lineages, strongly supporting their progenitor cell nature. Over-expression of representative mesenchymal stem cell makers for HCCs compared with NCs further distinguish them from NCs, as a progenitor cell population, which is consistent with other work regarding characterization of progenitor cells within articular cartilage or other tissues.

In addition, chemokine involved in progenitor cell and leukocyte recruitment were significantly up-regulated in HCCs as compared to NCs. CXCL-12 showed much stronger band in agarose gel electrophoresis, which may indicate the ability of HCCs to attract more progenitor cells or inflammatory cells such as leukocytes and macrophages when activated (by isolation, injury, inflammatory stimuli, *etc.*). The ability of HCCs to secrete CXCL-12 may enable them to recruit more endogenous progenitor cells within articular joint upon injuries[29], and also facilitate the attraction of inflammatory regulator cells[30] to clean up necrotic cells and damaged tissue, thereby expediting the process of tissue repair.

In vitro chemotaxis assays by HMGB-1, a nuclear protein, previously identified as core chemoattractant post-traumatic cartilage injury[24], revealed that HCCs were more active in migration than NCs upon stimulation. This was consistent with previous study that CPCs have migratory capability, and respond rigorously towards focal injury after cartilage impact. This might also indicate that HCCs can be attracted by damage associated chemotactic factors for tissue repair and regeneration.

Multilineage differentiation, along with colony-forming ability and surface marker expression, is one of the defining characteristics of mesenchymal stem cell populations. Therefore, our study evaluated HCCs multipotency in classic differentiation culture system. We found that HCCs can be readily induced toward chondrogenic and osteogenic differentiation. However, they have very limited adipogenic ability. This result is consistent with published data for cartilage progenitor cells, as well as CPCs in our previous study[24]. Therefore, HCCs are not like mesenchymal stem cells, which are multi-potent and are able to differentiate towards three-lineages. Instead HCCs are more likely to be progenitor cells, which have lost some of the “stemness” and appear later along the lineage commitment path. They appear to reside quietly in the articular cartilage matrix until they are activated by conditions, such as trauma and inflammation, to restore damaged cartilage tissue.

Although previous studies have pointed out that cartilage progenitor cells mainly reside on the superficial layer of immature bovine articular cartilage[18], our study demonstrates that HCCs can be isolated from both the superficial 1/3 and the deep 2/3 of articular cartilage, albeit the superficial 1/3 has significantly more progenitor cells than the deep 2/3. This difference may result from the distinct environment of the superficial vs the deep zone of articular cartilage. The superficial zone cells are subject to more shear stress and undergo rapid turnover[31], requiring more progenitor cells to replenish tissue loss[32], while the deep zone cells are subject to more compressive stress[33], and thus may quietly reside in the surrounding ECM with minimal tissue remodeling required. Progenitors from both layers show their bi-lineage differentiation potential for chondrogenesis and osteogenesis. Deep 2/3 progenitors showed stronger chondrogenic and osteogenic ability than superficial 1/3 progenitors, as well as higher progenitor cell transcription factors gene expression, which may indicate that these cells represent a more primitive population with less a differentiated phenotype and more plasticity to give rise to multiple cell types in osteochondral tissues, or it may indicate that during the process of tissue maturation early progenitors have differentiated again to produce distinct sub-populations specific for the different zones of articular cartilage. Their primary function could involve formation of neo-cartilage, as well bone tissue during the skeletal development. Moreover, upon injury or degradation of articular cartilage and/or sub-chondral bone, they could be called upon for tissue repair.

Our previous study showed that there exists a highly proliferative cell population (CPCs), which responds to cartilage superficial focal damage, and secretes lubricin, a protein for joint lubrication and cartilage surface maintenance[24]. These cells might come from the proliferation of progenitors identified in the superficial 1/3 in this study since we also showed in this study that progenitor cells from the superficial 1/3 has higher lubricin expression compared with those from deep 1/3. Such cells may normally proliferate only slowly and reside in their niche of cartilage ECM. When they are activated by traumatic injuries or other biochemical and/or mechanical alteration, they may actively proliferate and migrate towards injury sites for tissue repair. In addition, in the late stage OA, they might also present in the repair tissue. Here we also reveal that less differentiated population of cells resides in the deep 2/3 of the articular cartilage. Thus, the progenitors from the superficial 1/3 may actually originate from these deep 2/3 progenitors during the growth and development of articular cartilage, gradually gaining their more committed phenotype with specified functions like joint lubrication.

In conclusion, although articular cartilage is notorious for its poor healing ability posttraumatic injuries, our discovery of HCCs from a single cell cloned progenitor cell population, demonstrates the cartilage's intrinsic self-repairing potential. Our single cell sorting and clonogenicity screening successfully identify HCCs from both the superficial 1/3 and the deep 2/3. Further, they have different lineage specific gene expressions and distinct differentiation

potential, which may represent their distinct function in regard to cartilage repair.

Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

Conception and design: Yu, Zheng, Martin.
Collection and assembly of data: Yu.
Analysis and interpretation of the data: Yu, Zheng, Martin.
Drafting and revising of the article: Yu, Martin, Buckwalter.

Conflict of interests

The authors have no financial or personal relationships with entities that could have influenced this work.

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